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Down-regulation of SPARC/Osteonectin/BM-40 Expression in Methylcholanthrene-induced Fibrosarcomas and in Kirsten-MSV Transformed Fibroblasts

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SPARC (secreted protein acid and rich in cysteine), also known as osteonectin or BM-40, is a glycoprotein associated with the extracellular matrix of bone as well as with many soft tissues that produce extracellular matrix, including matrix-producing tumours. Northern and slot-blot analyses were used to study SPARC expression in tumours induced *in vivo* by methylcholanthrene (MCA) and in transformed cells induced *in vitro* by Kirsten-MSV and SV-40 infection. MCA-induced tumours expressed SPARC mRNA at quantitatively different levels. Fibroblasts transformed *in vitro* by Kirsten-MSV, and, to a lesser extent, by SV-40, showed reduced levels of SPARC mRNA expression compared with normal fibroblasts. Run-on assay indicated that transcription of SPARC was lower in the Kirsten-MSV transformed cells than in the normal parental fibroblast culture. However, SPARC mRNA in the transformed culture was as stable as that in normal culture. The difference, therefore, between levels of SPARC mRNA in transformed and normal culture was mainly due to different rates of transcription. Cloned cell lines derived from the Kirsten-MSV transformed culture also showed heterogeneous expression of SPARC: two lines had high and two had low expression of the gene. The level of mRNA correlated with that of the protein secreted. The SPARC expression might contribute to the malignant phenotype.
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INTRODUCTION

SPARC (secreted protein acid and rich in cysteine), osteonectin and BM-40, isolated and named independently, are the same secreted 43 kD calcium-binding glycoprotein [1–3]. This protein constitutes about 25% of the non-collagenous protein in fetal calf bone [4] and binds hydroxyapatite and type I collagen with high affinity [5]. cDNA probing has shown that other soft tissues and cells also express this gene. Many of these SPARC expressing cells also produce extracellular matrix proteins. These include Sertoli cells of the testis, cumulus oophorus cells of the ovary and stroma cells and keratinocytes of the skin [6–8]. Tumour cells such as osteosarcomas [9], carcinomas [10] and melanoma [11] have also been shown to express the protein.

Despite several studies on the distibution of SPARC mRNA in various tissues and cells and its association with extracellular matrix-producing cells, SPARC was found in only some of the matrix of SPARC producers. SPARC is present, for instance, in the basement membranes of decidual cells of human endometrium but not in the uterine glandular epithelium [10]. In Reichert's membrane, results are contradictory [2, 12]. Similarly, even though SPARC might play a role in proliferation of cells by interacting with cells and the extracellular matrix, causing their separation, this role is functional in only some SPARC producers [13].

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Transformation of cells by viruses or viral oncogenes affects the complex expression of SPARC. Transformation of NIH-3T3 cells by viral oncogenes such as v-*abl* or v-*src*, or of chick embryo fibroblasts by Rous sarcoma virus, down-regulates SPARC expression [7, 14]. However, transformation with SV-40 has no such effect [7, 11]. In addition, examining a panel of melanoma cell lines, one of us (C.C.H.) found a heterogeneous expression of SPARC [11], indicating that the level of expression was characteristic of individual cells. We now report SPARC expression in a panel of fibrosarcomas induced by chemical carcinogen and in fibroblasts transformed by Kirsten-MSV.

MATERIALS AND METHODS

Mice. Female BALB/c, DBA/2 and (C × B6) F1 mice aged 8–10 weeks were purchased from Charles River.

Tumours. All tumours, except the colon adenocarcinoma C-26, were induced in our laboratory by subcutaneous injection of 100 μg methylcholanthrene (MCA) (Eastman Organic Chemicals) and maintained *in vivo* by subcutaneous passages in syngeneic mice. The MCA induced tumours were histologically characterised as fibrosarcomas.

RNA analysis. Total cellular RNA was isolated from cells or frozen tissues by the guanidine isothiocyanate/CsCl method [15], run in 20 μg aliquots on 1% agarose/formaldehyde gels,

transferred to nylon filters (Hybond/N, Amersham) and bound by ultraviolet crosslinking. Nylon filters were hybridised with ^{32}P-labelled probe (2×10^6 counts per min (cpm) per ml) in 10% dextran sulphate, 50% formamide, 1 × Denhardt's solution and competing DNA and RNA as described [16] at 42°C for 18 h. Hybridised filters were washed at high stringency (0.1 × sodium saline citrate, 0.1% sodium dodecylsulphate (SDS) at 65°C) and exposed to Kodak XAR-5 films with Du Pont Lighting Plus intensifying screen at −70°C. RNA slot blots were done in a Manifold II (Schleicher & Schuell); starting from 20 μg, three 10-fold dilutions were loaded for each sample. RNA molecular weights were estimated with a (BRL) RNA ladder as standard. Densitometric analysis was done with an LKB Ultrascan.

In vitro nuclear transcription and actinomycin D treatment. Isolation of nuclei, *in vitro* transcription in the isolated nuclei and hybridization of the transcribed RNA to p2-4 cDNA immobilised on a nylon filter were done as described [17]. Actinomycin D (Merck, Sharp & Dhome) was added to cultured cells at 10 μg/ml and left for specified times before the cells were processed for RNA extraction.

Probes. The following probes were used: a 800 basepair (bp) *Hind*III-*Bam*HI insert of p2-4 cDNA containing SPARC sequences [6], a 500 bp *Eco*RI fragment of p-rlf-1 containing rat fibronectin cDNA [18], a 750 bp *Pst*I fragment of pOD48 plasmid containing ornithine decarboxylase (ODC) cDNA [19], a 800 bp *Pst*I insert of plasmid LL Rep 3 containing mouse repetitive sequences isolated from a liver cDNA library [20] and a 1.6 kb fragment of pABE plasmid containing human 28S rRNA sequences [21].

Infection with retroviruses. Amphotropic virus stocks were obtained by superinfection of a Kirsten-MSV non-producer transformant clone of NIH-3T3 [22] with supernatant from NIH3T3 cells carrying 4070 A amphotropic MuLV [23]. The normal fibroblast culture was derived from muscle of newborn BALB/c mice, which was minced and disaggregated in 0.25% trypsin (Difco). The freshly derived BALB/c fibroblasts (2×10^6) were incubated with a 1:5 dilution of virus stock and 8 μg/ml polybrene for 48 h, and then washed and screened for development of transformed foci. Transformed foci were pooled, grown to confluence and tested for tumorigenicity in syngeneic BALB/c mice. The mice were infected with SV-40 virus (a gift from Dr Barbara Knowles, Wistar Institute, Philadelphia) as described [24].

Immunoprecipitation. Confluent cultures of SN13 and SN14 lines were labelled overnight with 1.85 MBq ^{35}S-methionine (Amersham, over 40.7 CiBq mol). The labelled culture medium was collected and about 10^7 trichloro-acetic acid precipitable cpm from each of the two culture media was immunoprecipitated in the presence of protease inhibitor α-toluenesulphonyl fluoride (PMSF) by incubating media with 20 μl of an antiserum to SPARC at room temperature for 30 min and then with 50 μl protein A–Sepharose CL-4B (50% suspension, Pharmacia) at 4°C for 1.5 h. The immunoprecipitates were washed three times with NET buffer (1% NP-40, 0.4 mol/l NaCl, 50 mmol/l Tris, pH 8.0, 5 mmol/l EDTA, 1 mmol/l PMSF), once with 10 mmol/l Tris, pH 6.8, eluted with electrophoresis sample buffer at 100°C for 10 min and then analysed by SDS–polyacrylamide gels in the presence of dithiothreitol [25].

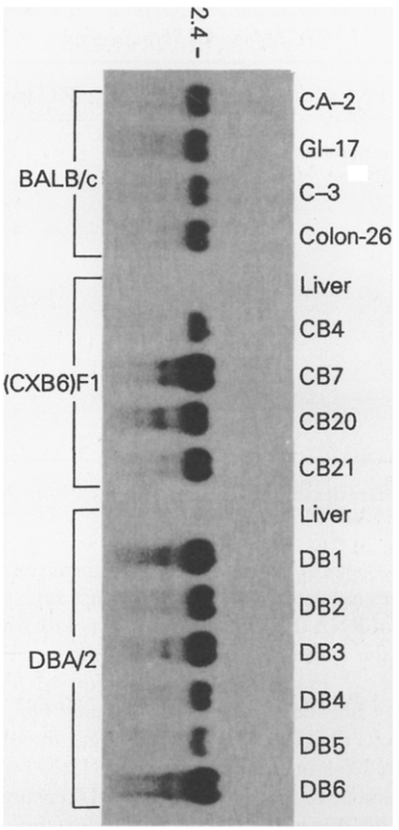

Fig. 1. Northern blot of total tissue RNA from mouse tumours and normal liver hybridised with SPARC probe. All tumours are fibrosarcomas except colon-26, which is a colon adenocarcinoma.

RESULTS

Osteonectin expression in MCA-induced fibrosarcomas

To investigate the effects of chemical carcinogenesis on SPARC expression, we generated in mice of different genetic background a panel of fibrosarcomas with MCA. Northern hybridisation showed that SPARC RNA was expressed in each of the induced tumours. However, the level of expression varied among different tumours independently of the genetic background (Fig. 1). Comparison of these fibrosarcomas with

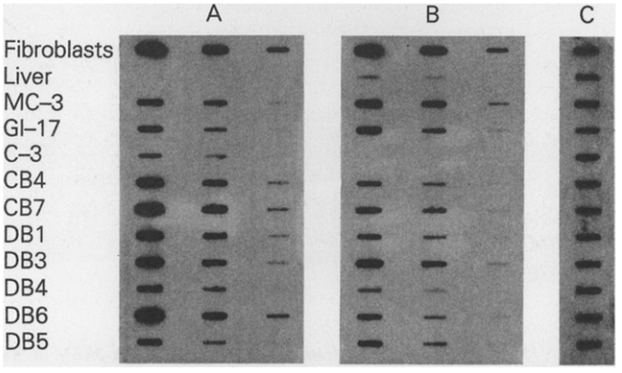

Fig. 2. Slot blot of RNA for normal fibroblasts and MCA-induced mouse fibrosarcomas hybridised with (A) SPARC, (B) fibronectin and (C) housekeeping gene LL Rep 3 probes.

Table 1. *SPARC and fibronectin RNA expression in MCA-induced fibrosarcomas*

	SPARC	Fibronectin
Normal fibroblasts	100*	100
Normal liver	2.1	13.9
MC-3	27.2	59.3
G-17	19.9	46.0
C-3	11.3	2.5
CB4	46.0	25.3
CB7	62.3	37.6
DB1	41.7	28.4
DB3	40.7	48.4
DB4	19.6	19.3
DB6	68.0	33.3
DB5	15.0	37.4

*Arbitrary densitometric units (%).

the early passages of normal fibroblasts from normal newborn mouse tissues indicated that all the tumours analysed expressed less SPARC mRNA than normal fibroblasts with some variations in level of the transcript among the tumours (Fig. 2A). For comparison, we examined the expression of fibronectin by hybridising a fibronectin probe to the same blot. Fibronectin expression was not only down-regulated in tumour cells, as has been reported for other neoplastic cells [26], but also varied from one tumour to another (Fig. 2B). To ensure that similar amounts of RNA were loaded onto each slot, the same blot was hybridised to the cDNA copy of a housekeeping gene (LLRep 3). We did not detect significant differences in the loaded RNAs (Fig. 2C). A quantitative estimation of SPARC and fibronectin expression in tumours, with normal fibroblast expression arbitrarily defined as 100%, is shown in Table 1.

SPARC expression in Kirsten-MSV induced transformants

Since SPARC expression has been reported to be down-regulated in fibroblasts transformed by RNA tumour viruses or their oncogenes [7, 14], we tested whether other RNA and even DNA tumour viruses can cause this effect. We infected a freshly derived normal BALB/c fibroblast culture (4th passage) with SV40 or Kirsten-MSV viruses and examined the levels of SPARC RNA in the transformed cells. Slot-blot analysis showed that the Kirsten-MSV transformed (KRT) and the SV40-transformed culture had a reduced level of expression compared with the untransformed parental fibroblast culture (Fig. 3A), but effective suppression of SPARC occurred in KRT cells only. Densitometric analysis of the slot blot of Fig. 3A indicated that KRT and SV-40 transformed fibroblasts expressed 50% and 15% less SPARC RNA than normal fibroblasts, respectively. A run-on assay was used to determine whether the differences in the levels of mRNA were due to differences in the rate of transcription. This assay predominantly measures the elongation of *in vivo* initiated RNA chains and thus estimates the relative number of RNA polymerases on a particular gene at the time nuclei are harvested. Figure 4 shows that in the KRT culture the SPARC gene was less actively transcribed than in normal parental cells. Values of the SPARC RNA transcript (arbitrary densitometric units) in normal and KRT fibroblasts were normalised to the amount of ribosomal RNA transcription. SPARC transcript in KRT cells was 70% less than that in normal fibroblasts. Message stability was also examined in the normal and the KRT culture that had been treated with actinomycin D to inhibit transcription. Analysis of RNA indicated that the SPARC mRNA levels remained the same for both the normal and transformed cultures even after 8 h of actinomycin D treatment (Fig. 5A). Thus the difference in the level of SPARC mRNA in the transformed vs. normal fibroblasts was due to differences in transcriptional rate rather than mRNA stability. Since the ODC transcript has a short half-life in normal fibroblasts [27], although it becomes stable in transformed fibroblasts [28], the same blot was hybridised to an ODC cDNA probe as a control. Figure 5B shows the expected expression pattern for the ODC gene in our normal and KRT transformed fibroblasts.

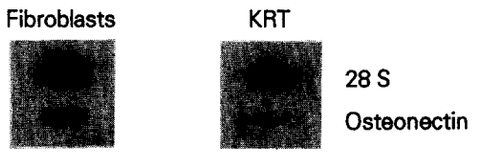

Fig. 4. *In vitro* transcription in isolated nuclei. Transcript synthesised from normal fibroblast nuclei were hybridised to excess denaturated SPARC cDNA or cDNA copy of 28S ribosomal RNA.

SPARC expression in clonally derived KRT transformants

The original KRT fibroblast culture was passaged *in vivo* in syngeneic BALB/c mice to ensure that the cells were tumorigenic. The resulting tumour was then isolated and cultured *in vitro* under limited dilution (0.3 cells per well) to obtain clonally derived cell lines. When four clones were tested for SPARC mRNA expression, not all had the same reduced level of expression (Fig. 3B). Clones 6 and 13 expressed more SPARC

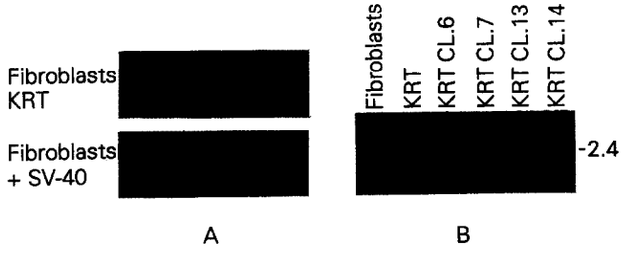

Fig. 3. (A) Slot blot of RNA from normal and Kirsten-MSV or SV-40 virus-transformed fibroblasts. (B) Northern blot of total cellular RNA obtained from normal fibroblast culture, Kirsten-MSV transformed fibroblast culture and cell lines 6, 7, 13 and 14 clonally derived from the transformed culture.

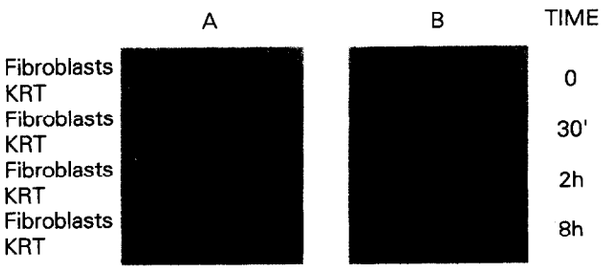

Fig. 5. Slot blot of RNA from normal and KRT fibroblast culture treated with actinomycin D. Slot blot was hybridised with (A) SPARC and (B) ODC probes.

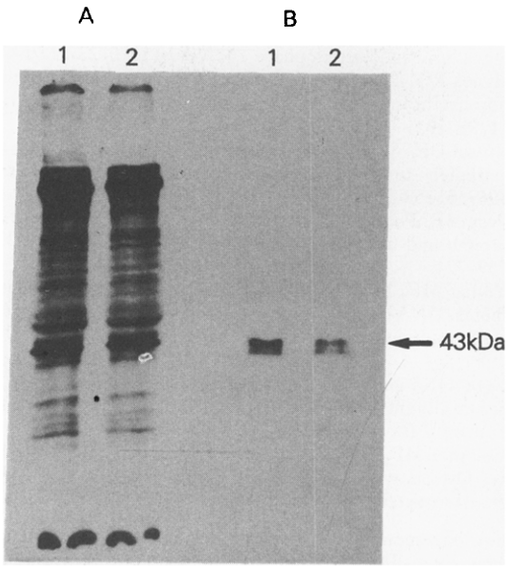

Fig. 6. Synthesis of SPARC by cell lines clonally derived from KRT culture. Cell lines 13 and 14 were labelled with ^{35}S methionine, and SPARC protein from cytoplasmic lysate (A) or secreted into culture medium (B) was immunoprecipitated and analysed by SDS–polyacrylamide gel electrophoresis. Lanes 1 and 2 from clones 13 and 14, respectively.

mRNA than 7 and 14. Thus, as in MCA-induced tumours, SPARC mRNA expression in the cloned KRT cell lines was heterogeneous. Analysis of cytoplasmic lysates and of the culture media of clones 13 and 14 showed that the transcript was translated and the product secreted into the culture medium. Furthermore, the level of the protein secreted correlated with that of the mRNA in the cells (Fig. 6B).

DISCUSSION

We investigated the effects of neoplastic transformation on SPARC expression. Since the characteristics of established cell lines, such as NIH-3T3 or chick embryo fibroblasts, used in previous studies are known to deviate from those of normal fibroblasts, we derived a fresh normal fibroblast line from BALB/c tissues. Transformation of this normal cell culture with Kirsten-MSV resulted in down-regulation of SPARC gene expression. Thus, the result of transformation of our normal fibroblasts is in agreement with the results of transformation of established cell lines with other RNA tumour viruses [7, 14]. Transformation of the same fibroblast culture with the DNA tumour virus SV-40 had no effect on the expression of SPARC mRNA. We also derived some clonal cell lines from the KRT culture: expression among these cell lines was heterogeneous in that not all the cloned lines had the same reduced level of expression.

Similarly, SPARC expression was not uniformly supressed in all fibrosarcomas induced by MCA. In a previous study, one of us (C.C.H.) found a similar heterogenous expression pattern among primary and metastatic melanoma cell lines [11]. Together, these results indicate that tumours, whether occurring naturally or induced by viruses or chemical carcinogens, have a heterogenous cell population even for the pattern of SPARC expression. This interpretation was further strengthened by the observation that among the MCA-induced fibrosarcomas, the expression of fibronectin was also differently suppressed.

SPARC binds to cell surface and various collagen types in a calcium-dependent manner and mediates dissociation of cells from an extracellular matrix. Study of osteonectin promoter indicated some analogy among osteonectin, type I collagen and alkaline phosphatase genes, which are highly expressed in bone cells [29]. In particular, osteonectin and type I collagen may share a common mechanism for gene activation [29] and it would be of interest to see whether type I collagen expression in tumours correlates with that of SPARC. It has also been suggested that SPARC influences cell migration and proliferation [13, 30]; as a tumour basement membrane and extracellular basement component, SPARC may affect adhesion among tumour cells (thus preventing tumour spread) or favour colonisation when tumour cells are already circulating in blood vessels. Thus, the level of SPARC expression among transformed cell lines may correlate with tumorigenicity. Our preliminary results suggested that high SPARC expression is associated with high lung colonisation [31].

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Chemoresistance in Rat Ovarian Tumours

W.J. Zeller, S. Frühauf, G. Chen, B.K. Keppler, E. Frei and M. Kaufmann

In a cisplatin resistant subline (O-342/DPP) of an intraperitoneally growing transplantable rat ovarian tumour (O-342), intracellular glutathione (GSH) was approximately doubled (mean [S.E.] 1.5 [0.26] vs. 0.8 [0.2] nmol/10^6 cells). GSH reductase activity was higher (30.64 [4.07] vs. 20 [0.92] nmol/min per mg protein), although no difference was found for GSH-S-transferase. 24 h after exposure to cisplatin, formation of DNA interstrand cross-links was at a maximum in both lines and significantly higher in O-342 (162 [23] vs. 88 [22] rad eq). Combination treatment of O-342/DDP with buthionine sulphoximine plus cisplatin resulted in a marginal increase in survival compared with cisplatin treatment; treatment of this line with 3-aminobenzamide plus cisplatin was also superior to cisplatin alone. In the sensitive line both combinations were likewise superior to cisplatin alone. *In vitro*, at equimolar concentration, a new platinum complex (CTDP) was at least as active as cisplatin in both lines, which suggests a superior therapeutic index because its LD_{50} in mice is threefold higher than that of cisplatin. A ruthenium complex (ICR) had a higher activity in the resistant line. A titanium complex (budotitane) was not active.
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INTRODUCTION

MANAGEMENT OF ovarian cancer has been improved by the introduction of cisplatin although the development of resistance limits its use. To find new drugs that are superior to cisplatin or are active in cisplatin-resistant tumours, a series of new metal compounds was developed by one of us (B.K.K.), including complexes of platinum, titanium, zirconium, hafnium or ruthenium [1–4]. These compounds were tested in different experimental tumour models, among them autochthonous mammary and colorectal cancer in the rat, and some have shown promising results. To have an appropriate ovarian tumour model for further evaluation of these drugs and to study resistance mechanisms in experimental ovarian tumours, we are establishing a panel of such models, including animal and human tumour lines. One model, rat tumour O-342 and its cisplatin-resistant subline, O-342/DPP, is described here. Glutathione (GSH) modulation by buthionine sulphoximine and modulation of DNA repair by 3-

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